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Molecular Confirmation of Host Records for Ichneumonoid Parasitoids of Wood-boring Beetle Larvae

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Abstract.—Field observations in Sabah of a large braconine wasp, *Shelfordia* sp., investigating and ovipositor-probing at a frass hole in an exposed tree root suggested the possible location of a host. Investigation of the substrate revealed a paralysed and partly consumed larva of an anthribid beetle that had a 1st instar parasitic wasp larva on it. Both adult and larval wasps were sequenced, in separate laboratories, for the D2-D3 expansion region of the nuclear 28S rDNA gene. The sequences were identical and consideration of their unique features compared with many other sequences from ichneumonoids leads to confirmation that the hosts of *Shelfordia* spp. include Anthribidae living in tree roots. The implications of DNA sequencing for constructing quantitative food webs are discussed.

Host records for parasitic Hymenoptera are notoriously unreliable as has been well documented by Shaw (1994) and Noyes (1994). Errors in the literature derive from several sources including misidentification of the parasitoid, misidentification of the host, misidentification of both, and through the wrong association of a parasitoid with a putative host because of contamination of the rearing system. These problems are particularly true for concealed hosts such as wood-borers, gall makers, etc., because these substrates can contain many potential host species apart from the one that may be the focus of attention. Simply rearing a parasitoid and a potential host from a given piece of substrate has consequently often led to incorrect conclusions about what is parasitising what. Although it is sometimes possible carefully to dissect substrate and to identify any host and parasitoid remains, or to isolate and rear the host and its parasitoid in isolation, these are both difficult procedures requiring much skill and at least some luck. The development of molecular techniques, in particular DNA sequencing, has opened new and in some respects easier ways to solve these problems.

The braconine genus *Shelfordia* Cameron is relatively common from India, through the Indo-Australian Region, to N.E. Australia, often being found in local collections, no doubt because its species are rather large by parasitic wasp standards. Its identification has not been without problems though, and since its original description (Cameron 1902), nothing had been published on it under that name until it was discovered to be a senior synonym of *Sigalphogastra* Cameron (Quicke 1982). The genus name *Sigalphogastra* had, however, been very inconsistently applied, and few of the older publications citing this name (Shenefelt 1978) actually refer to taxa congeneric with the type species. In 1984, Quicke described a new genus *Rostraulax*, based on a species similar

to *Shelfordia sensu stricto*, but which had an elongate labio-maxillary complex and partly fused apical flagellomeres (Quicke 1984a). Although *Rostraulax* is recognisable by these autapomorphies, *Shelfordia* is, as far as we can tell at present, left paraphyletic with respect to *Rostraulax*, and the letter was formally synonymised with *Shelfordia* by van Achterberg (1993). Prior to now, there have been no available host data for the genus *Shelfordia*, although the especially long ovipositor of one Indian species, *S. longicaudata* van Achterberg, suggests that it is potentially a parasitoid of a host living deeply within wood (van Achterberg 1993). Since its original description, some 35 species have been reclassified into it (or its synonym, *Rostraulax*) (Quicke 1983, 1984b, 1985, 1988, 1991, Quicke and van Achterberg 1990, Quicke and Koch 1990, van Achterberg 1993, van Achterberg and O'Toole 1993), of which 11 are from the island of Borneo, all from Sarawak. No species-level keys are available for *Shelfordia*, and proper taxonomic revision is required before the species referred to here can be properly identified.

FIELD OBSERVATIONS

In August 1999, DLJQ and NML had the opportunity to collaborate with the group of Dr Maryati Mohamed of the Tropical Biodiversity and Conservation Unit, Universiti Malaysia, Sabah, and to observe some large braconid wasps in lowland tropical rain forest at Poring (Kinabalu National Park). Along a short stretch of one popular forest trail, some six or seven conspecific females of the large, entirely tropical, braconine wasp genus *Shelfordia*, were seen flying or sitting on low vegetation over a period of several days. As no host records are known for this genus we decided to observe them to see what they were attacking. Large braconine wasps are typically thought to be parasitoids of wood-boring beetle or lepidopteran hosts, and a large dead tree with abundant signs of borer activity nearby seemed to be the obvious place for them to be searching. However, after several hours of observation, the wasps were never seen near this tree, but only near the path. The wasps were very cautious; they remained stationary on the low vegetation for long periods, and were easily disturbed by tourists passing by. After some while, however, we observed one and then another fly to land on a small (approximately 5cm diameter) tree root that was partially exposed by the path. Again the wasps remained stationary for about 20 minutes when one of them approached a boring from which fresh-looking wood particles were exuded. After antennating the site for several minutes, she raised her metasoma and probed into the frass hole with her ovipositor and sheathes. After a few moments she was observed making marked twisting movements with the apex of her metasoma and she was then collected.

METHODS

Collection.—The apparently-ovipositing adult female *Shelfordia* was collected with a net and placed into a clean vial containing 95% ethanol. Other adult wasps referred to in this paper were collected in the same way or in Malaise traps containing 95% ethanol. The tree root with frass hole was dissected the same day with a saw, hammer and chisel, and a boring located below the frass hole which contained a paralysed and partly deflated beetle larva upon which was a single small (<1mm long) parasitoid larva. The beetle and parasitoid larvae were handled with watch-makers' forceps and transferred to a clean tube with 100% ethanol.

Laboratory protocols.—For adult insects, DNA was extracted in the U.K. from single individuals stored in 100% ethanol by incubation at 37°C in Proteinase K for approximately 18 hours, followed by sodium acetate/ethanol precipitation and re-suspension in 20µl TE buffer. PCR reactions were carried out in 50µl volumes containing 0.5µl DNA extract, 0.5µl Boehringer *Taq*, 1.25 µl 20µM primer, 1.25 µl 10mM

dNTPs and 5μl buffer. PCR conditions were 30 cycles of 98°C denaturation (15 seconds), 48°C annealing (30 seconds) and 72°C extension (40 seconds) with an initial denaturation of 3 minutes at 93°C and a final extension of 3 minutes. PCR products were purified with Pharmacia Amersham PCR purification kit and then sequenced directly using *Amplitaq FS* on an ABI 373 automated sequencer.

The parasitoid larva was stored in 95% ethanol and DNA extraction and sequencing was carried out in Helsinki. The larva was dried and ground in 50μl TNE-buffer (1 M Tris, 5 M NaCl, 0.5 M EDTA) and 1μl proteinase K (Sigma Chemical Co.). The samples were then incubated at 37°C overnight, followed by sodium acetate and ethanol precipitation, and resuspension in 20 μl TE-buffer (as TNE but without NaCl). PCR reactions were carried out in 50 μl volume reactions, 5 μl 10× Buffer II (Perkin-Elmer), 5 μl of 25 nM $MgCl_2$, 2 μl 10 μM primer, 3 μl of 10 nM dNTP, 0.25 μl *Amplitaq* DNA Polymerase, 31.75 μl H_2O and 1 μl DNA template. PCR conditions after an initial denaturation at 94°C (2 min), were denaturation at 96°C (15 s), annealing at 55°C (30 s), extension at 72°C (1 min) for 35 cycles and a final extension 72°C (7 min).

The larval PCR product was purified using Nucleospin PCR Purification kit and then sequenced in both directions using Big Dye terminators on an automatic sequencer ABI PRISM 377 (Perkin-Elmer).

The beetle larva was identified as an anthribid by reference to Lawrence and Britton (1991). Specimens are deposited in The Natural History Museum, London. Full DNA sequences are in the EMBL/GenBank/DDBJ databases; accession numbers AJ231540, AJ231541 and AJ277499-AJ277504.

RESULTS

The parasitoid larva and the putatively conspecific *Shelfordia* adult produced identical sequences for the 480 base pair length of the D2 region that was readable for both (the small larva gave a weak sequence). These sequences were aligned by eye to each other and to those of a range of other S.E. Asian Braconinae, including a similarly-sized species of the related genus *Diamblomera* Enderlein, collected in a similar site approximately 150 meters away (Quicke *et al.* 2000), and several other braconines from Poring. Part of the alignment is shown in Figure 1, in which several features that are shared by both the female *Shelfordia* and the parasitoid larva found are indicated in bold italics.

DISCUSSION

The present findings of an identical 28S D2 DNA sequence for both the adult *Shelfordia* and the parasitic wasp larva found on its anthribid host (Fig. 1) can only be considered in the light of our knowledge of interspecific variation of this gene region within the Braconidae, and more precisely, the Braconinae. In the laboratory at Silwood Park, this gene region has been sequenced for more than 250 species of Braconidae and for 98 species of Braconinae (Belshaw *et al.* 1998, in preparation). On no occasion were any two species found to have identical sequences, even congeneric species always differing by at least two or three bases, and often by the presence of species-specific insertions or deletions (the above-mentioned Braconinae data set includes 7 species each of *Digonogastra* Ashmead and of *Bracon* Fabricius). Note also that there are several differences between the sequences of the two *Nesaulax* Roman species from Sabah sequenced (Fig. 1). Contamination of the samples is considered highly improbable because the specimens were not handled by the same equipment, were placed in separate clean vials, and DNA extraction and sequencing was carried out in different countries. This study emphasises the potential for the use of DNA sequence technology for making firm parasitoid/host associations that would not otherwise

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ShelfordiaAD  TAATTGTAAGATGTTGTCGGCGTGCACTTCTCCCCTAGTAGGACGTCGCGACCCGT
ShelfordiaLA  TAATTGTAAGATGTTGTCGGCGTGCACTTCTCCCCTAGTAGGACGTCGCGACCCGT
DiamblomeraS  TAATTGTAAGATGTTGTCGGCGTGCACTTCTCCCCTAGTAGGACGTCGCGACCCGT
Nesaulax sp 1 TAATTGTAAGATGTTGTCGGCGTGCACTTCTCCCCTAGTAGGACGTCGCGACCCGT
Nesaulax sp 2 TAATTGTAAGATGTTGTCGGCGTGCACTTCTCCCCTAGTAGGACGTCGCGACCCGT
Pachybracon   TAATTGTAAGATGTTGTCGGCGTGCACTTCTCCCCTAGTAGGACGTCGCGACCCGT
Nedinoschiza  TAATTGTAAGATGTTGTCGGCGTGCACTTCTCCCCTAGTAGGACGTCGCGACCCGT
Cratobracon   TAATTGTAAGATGTTGTCGGCGTGCACTTCTCCCCTAGTAGGACGTCGCGACCCGT
Hybogaster    TAATTGTAAGATGTTGTCGGCGTGCAcTTCTCCCCTAGTAGGACGTCGCGACCCGT

ShelfordiaAD  TGAGT*TTTTT*TGTT--GGTCTACGGCCCAAGTGGAAGCTTTTAAT*A*AATT------
ShelfordiaLA  TGAGT*TTTTT*TGTT--GGTCTACGGCCCAAGTGGAAGCTTTTAAT*A*AATT------
DiamblomeraS  TGAAT------GTT--GGTCTACGGCCCAAGTGGTAGCTTTTAGTGAATT------
Nesaulax sp 1 TGAGT------GTTTCGGTCTACGGCCCAAGTGGAAGCTTTTAATGAATT------
Nesaulax sp 2 TGAGT------GTTTCGGTCTACGGCCCAAGTGGAAGCTTTTAATGAATG-AATT-
Pachybracon   TGAGT-----TGTT--GGTCTACGGCCCAAGTGGGAGCTTTTAATGAATT------
Nedinoschiza  TGAGT------GTT--GGTCTACGGCCCAAGTGGAAGCTTTTAGTGAATGAAATTT
Cratobracon   TGAGTAC----GTT--GGTCTACGGCCCAAGTGGTAGCTTTTAATGAATA------
Hybogaster    TGAGT-----TGTT--GGTCTACGGCCCAAGTGGGAGcTTTTAATGAATT------

ShelfordiaAD  TTATTTATT*A*AAAA-CCCTTGGTGTT---TCCTGACTGGCACTCGTCGGTAT*A*T*A*C
ShelfordiaLA  TTATTTATT*A*AAAA-CCCTyGGTGTT---TCCTGACTGGCACTCGTCGGTAT*A*T*A*C
DiamblomeraS  TTATTCATTGAAAA-CCCTTGGTGTT---TCCTGACTGGCATTCGTCGGTAT-T-C
Nesaulax sp 1 TTATTTATTGAAAA-CCCTTGGTGTT---TCCTGACTGGCACTCGTCGGTAT-T-C
Nesaulax sp 2 TTATTTGTTGAAAA-CCCTTGGTGTT---TCCTGACTGGCACTCGTCGGTAA-T-C
Pachybracon   TTATTCATTGAAAA-CCCTTGGTGTT---ACCTGACTGGCACTCGTCGGTAT-T-C
Nedinoschiza  TTATTCATTGAAAA-CCCTTGGTGTT---TCCTGACTGGCACTCGTCGGTAT-T-C
Cratobracon   TTATTCATTGAAAA-CCCTTGGTGTTGTTTCCTGACTGGCACTCGTCGGTAT-T-C
Hybogaster    TTATTCATTGAAAAACCcTTGGTGTT---TCCTGACTGGCGCTCGTCGGTAT-T-C

ShelfordiaAD  *A*TATGGTATTGA---GCCGCAT-TA-ATTA----TATGCGTCTAT--ATCTGTCGC
ShelfordiaLA  *A*TATGGTATTGA---GCCGCAT-TA-ATknnnnnnnnnnnnnnnnnnnnnnnnnnn
DiamblomeraS  GTATGGTATTGA---GCCGCAT----ATTA----TATGCGTTCAT--ATCTGTCGC
Nesaulax sp 1 GTATGGTATTGATCAGCCGCAT----ATTA----TATGCGTTCGT--ATCTGTCGC
Nesaulax sp 2 GTATGGTATTGA---GCCGCAT----ATTA----TATGCGTCCAAATATCTGTCGC
Pachybracon   GTATGGTATTGA---GCCGCATAT--ATTA----TATGCGTCCAT--ATCTGTCGC
Nedinoschiza  GTATGGTATTGA---GCCGCATATATATTA----TATGCGTTCAT--ATCTGTCGC
Cratobracon   GTATGGTATTGA---GCCGCA-----ATTATATATATGCGTCCAT--ATCTGTCGC
Hybogaster    GTATGGTATTGA---GCCGCAC----ATTA---ATATGCGTCCTT--GTCTATCGC
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Fig. 1. Manually aligned 28S D2 rDNA sequences including adult of the *Shelfordia* found probing the anthribid burrow (ShelfordiaAD), the larva found on the anthribid beetle larva (ShelfordiaLA), other braconines from Poring (*Diamblomera* sp., *Nesaulax* spp 1 and 2 and *Pachybracon* sp.), and other large braconine wasps from the Island of Borneo. Bases unique to the *Shelfordia* female and the putatively conspecific parasitoid larva are indicated in ***bold italics***.

be possible. Although DNA-based techniques have been used to detect parasitoid larvae inside their hosts (e.g., Greenstone and Edwards 1998), and similar results have been obtained recently for predatory insects using traces of prey DNA remaining in the predator's gut in a well characterised predator-prey system (Agusti *et al.* 1999), DNA has not yet been used 'blind' to determine hosts or prey in open systems. We show here that DNA sequence data, in combination with a data base of sequences of related taxa, has the potential to greatly facilitate studies not only on the autecologies of particular hosts through providing data on their parasitoids, but also to allow construction of fully quantitative food webs (e.g., Memmott and Godfray 1993). In this latter case, it also opens up the possibility of working on groups that are notoriously difficult to rear in isolation such as species that consume rotting wood and their parasitoids. With the addition of order-specific primers, it should also be possible to discover and identify endoparasitoids.

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